Onboard Sampling Protocol to Test the Efficiency of the AquaTriComb™ Ballast Water Treatment System developed by Aquaworx

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1. Introduction

Ballast water management systems have to meet the Ballast Water Performance Standard as stipulated in Regulation D-2 (see below) of the IMO Convention for the Control and Management of Ship`s Ballast Water and Sediments (hereafter referred to as the Convention). To challenge candidate treatment systems comprehensive and rigorous tests have to be undertaken using both land-based and shipboard trials. The test requirements are outlined in the IMO Guideline for Approval of Ballast Water Management Systems (G8).

G8 was primarily written as guideline for Administrations to assess whether or not ballast water treatment systems meet the standard as set forth in Regulation D-2 of the Convention. However, the guidelines can also be used as guidance for manufacturers and ship-owners when planning to undertake efficacy tests of such systems. Although shipboard tests are required in G8 as part of the approval process of a treatment system, the guidelines lack specific and detailed sampling protocols. This protocol intends to overcome this shortcoming.

The most up-to-date version of the Guideline G8 as agreed by IMO MEPC58 in October 2008 was used to prepare this sampling protocol.

The onboard tests of the ballast water treatment system developed by Aquaworx ATC GmbH (AquaTriComb^{TM} ballast water treatment system) will be undertaken in line with a Quality Assurance Project Plan (Annex 3) by GoConsult (see Annex 4) on the multi purpose vessel "Timbus", Rörd Braren Bereederungs GmbH & Co. KG, Kollmar, Germany (IMO No. 9198680, 4230 registered tonnage (BRZ), 6.489 DTW, length: 99,98 m, ballast water capacity 2557,9 m^3, 11 ballast water tanks). The vessel is fitted with one AquaTriComb^{TM} ballast water treatment system.

The required duration of the test period is at least 6 months and the first tests are planned to start in spring 2010. Two ballast tanks are identified for the tests, i.e. one tank for treated water and another for the control experiments. As required by the IMO the tests will be carried out during routine ship operations. While the timing of ship visits are not easily determined, the majority of the tests are likely to take place in the North and Baltic Seas.

1.1. IMO Ballast Water Discharge Standard

Regulation D-2 of the Convention stipulates that in order for ships to meet the requirements of the Convention these must discharge:

- Regulation D-2.1:
  - less than 10 viable organisms per cubic meter greater than or equal to 50 micrometers in minimum dimension, and
  - less than 10 viable organisms per millilitre less than 50 micrometers in minimum dimension and greater than or equal to 10 micrometers in minimum dimension, and
- Regulation D-2.2:
  - less than the following concentrations of indicator microbes, as a human health standard:
    - Toxigenic *Vibrio cholerae* (serotypes O1 and O139) with less than 1 Colony Forming Unit (cfu) per 100 millilitres or less than 1 cfu per 1 gramme (wet weight) of zooplankton samples,
    - *Escherichia coli* less than 250 cfu per 100 millilitres, and
    - Intestinal *Enterococci* less than 100 cfu per 100 millilitres.

As a consequence the treatment method must show a capability of reducing the viability of organisms to these standards according to their size. Further, the system must also address the indicator bacteriae as referred to in Regulation D-2.

2. Purpose of this protocol

The purpose of this sampling protocol is to prepare and produce a practical standard system for the shipboard efficacy tests of the ballast water treatment system. This protocol

- defines the test requirements for on-board efficacy tests, and
- describes the appropriate sampling strategies and procedures for such tests.

3. Challenge water

Standardised water conditions in shipboard tests as outlined in G8 address minimum numbers of organisms during ballast water uptake to "challenge" the treatment system under consideration. In addition abiotic water parameters need to be documented.

3.1. Abiotic water conditions

The source water for test cycles shall be characterized by measurement of salinity, temperature, particulate organic carbon and total suspended solids[1].

3.2. Organisms

Valid tests are indicated by uptake water, for both the control tank and ballast water to be treated, with viable organism concentrations exceeding 10 times the maximum permitted values in Regulation D-2.1 and control tank viable organism concentrations exceeding the values of Regulation D-2.1 on discharge[2].

This results in a minimum required organism density in the water to be treated and also for the control experiment during **uptake**:

- at least 90 viable organisms per cubic meter greater than or equal to 50 micrometers in minimum dimension, and
- at least 90 viable organisms per millilitre less than 50 micrometers in minimum dimension and greater than or equal to 10 micrometers in minimum dimension
- minimum concentrations of bacteriae are not required.

Upon **discharge** the number of organisms in the control tank water should exceed[3]:

- 10 viable organisms per cubic meter greater than or equal to 50 micrometers in minimum dimension, and
- 10 viable organisms per millilitre less than 50 micrometers in minimum dimension and greater than or equal to 10 micrometers in minimum dimension
- no limits for bacteriae.

[1] G8, Annex, Part 2, paragraph 2.2.2.9
[2] G8, Annex, Part 2, paragraph 2.2.2.5
[3] G8, Annex, Part 2, paragraph 2.2.2.5

4. Test cycles

The amount of ballast water pumped onboard in the test cycle should be consistent with the normal ballast operations of the ship and the ballast water management system should be operated at the treatment rated capacity for which it is intended to be approved.

A shipboard test cycle includes:

- the uptake of ballast water;
- the storage of ballast water onboard, and
- the discharge of ballast water from the ship.

4.1. Number of test cycles

Three consecutive valid test cycles which are to span a trial period of not less than six months[4] need to be performed (see Test Cycle Report, Annex 6). Any invalid test cycle does not affect the consecutive sequence[5].

The control and treatment test cycles may be run simultaneously or sequentially at each sampling event.

4.2. Validity of test cycles

A treatment test cycle should be deemed successful (= valid) if:

- uptake water, both the control tank and ballast water to be treated, contains viable organism concentration exceeding 10 times the maximum permitted values in Regulation D-2.1 and control tank viable organism concentrations exceed the values of Regulation D-2.1 on discharge[6].
- the average density of organisms greater than or equal to 50 micrometers in minimum dimension in the replicate samples of the treated water upon discharge from the ship is less than 10 viable organisms per cubic metre;
- the average density of organisms less than 50 micrometers and greater than or equal to 10 micrometers in minimum dimension in the replicate samples of the treated water upon discharge from the ship is less than 10 viable organisms per millilitre;
- the average density of *Vibrio cholerae* (serotypes O1 and O139) is less than 1 cfu per 100 millilitres, or less than 1 cfu per 1 gramme (wet weight) zooplankton samples upon discharge from the ship;
- the average density of *E. coli* in the replicate samples is less than 250 cfu per 100 millilitres upon discharge from the ship; and
- the average density of intestinal *Enterococci* in the replicate samples is less than 100 cfu per 100 millilitres upon discharge from the ship.

If in any test cycle the average discharge results from the control water is a concentration less than or equal to 10 times the values in Regulation D-2.1, the test cycle is invalid.

5. Sampling points

Sampling ballast *via* manholes and sounding or air-pipes is not used because these do not lead to representative sampling. Not all taxa are represented and densities of organisms provide misleading results when samples are taken in this way. Such sampling normally underestimates the densities of

[4] G8, Annex, Part 2, paragraph 2.2.2.7
[5] G8, Annex, Part 2, paragraph 2.2.2.8
[6] G8, Annex, Part 2, paragraph 2.2.2.5

organisms present. Consequently, samples should be taken at a sampling point along the ship's ballast water line as this provides direct access to the water.

Facilities or arrangements for sampling must be provided in the intake and discharge ballast water pipe of both the treated and control line to ensure that representative samples of treated and control water can be taken with as little adverse effects as possible on the organisms.

Treated water
Whenever possible, a ship should be selected enabling the installation of the candidate treatment system and its sampling points in a bypass allowing water flow with and without passage through the treatment system.

Sampling points should be installed into the ships ballast water piping system in a straight (at best horizontal) pipe section:
(a) prior the treatment system to evaluate whether untreated water samples attain the minimum density for organisms during intake[7], and
(b) as close as possible to the ballast water discharge point where the water exits the vessel to evaluate the treatment system performance, i.e. to proof whether or not the performance standard is met[8].

Control water
Control experiments should consist of water and organisms pumped through normal ship pipes that do not contain the treatment system.

Sampling points should be installed into the ships ballast water piping system in a straight (at best horizontal) pipe section:
(a) as close as possible to where the ballast water is taken up along the ballast water line and to determine the densities of organisms whether or not the minimum organism intake concentration is met, and
(b) as close as possible to the ballast water discharge point to sample for the density of surviving organisms in the untreated control water.

For water intake samples, the sampling points for treated and control water may be combined in case both tanks are filled simultaneously and the water is equally split after the sampling point into a control and treated line. At discharge separate sampling points for the control and treated line are essential. Consequently, a minimum of three sampling points are required.

Sampling points for both the control and treated water tests should be identical in design. Guideline G2 gives some guidance how a sampling point may be designed:

In order to undertake an accurate measurement on the organism concentration in the ballast water, it is recommended to install an "isokinetic" sampling facility. Isokinetic sampling is intended for the sampling of water mixtures with secondary immiscible phases (i.e. sand or oil) in which there are substantial density differentials. In such conditions, convergence and divergence from sampling ports is of significant concern. Since most organisms are relatively neutrally buoyant, true isokinetic sampling is unnecessary. However, the mathematics related to isokinetic sampling are deemed to be useful as a basis for describing and specifying sampling geometries. Isokinetic sampling is necessary to ensure that a sample contains the same proportions of the various flowing

[7] Where practicable the sampling point should be located prior the ships´ ballast water pump as the pump may also have an impact on organism survival.
[8] Regulation D-2 reads "… ships shall not discharge…", i.e. the closer the sampling is located to the ships discharge point the better.

constituents as the flow stream being sampled. During isokinetic sampling the sampling device does not alter the profile or velocity of the flowing stream at the moment or point at which the sample is separated from the main flow stream. Under isokinetic conditions, the velocities of both the sample and the main flow are equal at the point at which the sample is separated from the main flow. To achieve isokinetic sampling conditions, a sampler is designed to separate a subsection of the total flow-stream in a manner that does not encourage or discourage water entry other than that which is otherwise in the cross-section of the sampler opening. In other words, flow streams in the main flow of the pipe should not diverge or converge as they approach the opening of the sampler.

There are also further details in G8 regarding the isokinetic sampling point and it is recommend to consult G8 on this matter in detail before installation such sampling points onboard.

6. On-board sampling scenario

The following text describes the sampling scenario and sample analysis for onboard tests of ballast water treatment systems according to the IMO Guideline G8. In most of the following sections two sampling scenarios are described: (a) sample taking during the beginning middle and end of the ballast water pumping event and (b) sample taking during the entire ballast water pumping event (see Table 1 and Annex 1). However, for all samplings, scenario (b) will be followed as:

- Sampling over the entire pumping time results in most representative samples regarding organism concentrations. Scientific studies have shown that organisms are not homogenously distributed inside ballast water tanks, but that they concentrate in certain water layers. The location of the organism rich layers cannot be predicted as this depends on the holding time in the tank and ship movement influenced by weather conditions. Consequently, scenario (a) may result in missing the organism rich or poor water layers inside the tanks which may result in false positive samples.
- Scenario (a) results in up to 12 samples for the discharge sampling event of the control and treated water. In average it takes one hour to process one sample and the analysis of all samples should be completed within 6 hours after sampling[9]. Consequently, such a large number of samples cannot be processed onboard in time and for organisms above 50 micron in minimum dimension no other technology than working with living organisms, such as stains, is currently available.
- It should further be noted that to allow taking 12 samples of at least one cubic metre each 12 water collectors need to be installed onboard (see green bucket in Fig. 1a). This requires a foot print of at least 12 m² which is usually not available in engine rooms.

For the above mentioned reasons it is strongly recommended to follow sampling scenario (b). Of prime importance here is that this method will deliver the most representative results.

At best the control and water to be treated should be sampled in parallel during uptake and discharge. In case both tanks need to be operated consecutively two sampling events need to be undertaken during both uptake and discharge, i.e. one for the control and another for the water to be treated.

6.1. Samplings at ballast water uptake

The sampling for all organism groups should be undertaken in parallel, i.e. samples for all three organisms groups as outlined in Regulation D-2 should be taken at the same time.

[9] see 2.3.33 of G8

6.1.1. Abiotic water conditions

In addition to biological samplings, the source water for the test cycles shall be characterized by measurement of salinity, temperature, particulate organic carbon and total suspended solids. The water for these analysis purposes may be taken prior the sampling process for biological results is started. In minimum this should be done during ballast water uptake, but may also be considered during discharge.

6.1.2. Organisms above 50 micron in minimum dimension

Assuming the control and treated tank can be filled simultaneously, for organisms above 50 micron in minimum dimension at least 1 tonne of water needs to be sampled. This will be done over the entire uptake time (= at least 1 tonne of water) (see also Annex 1).

In case the control and treated tank cannot be filled simultaneously one sampling event is needed for each tank (control and treated), i.e. the above scenario needs to be carried out twice.

After sampling, the water sampled may be pumped back into the ships´ ballast water discharge line, dumped in the bilge water system or discarded otherwise.

Sampling such large volumes of water is a challenge. To be enabled to sample such large volumes of water the German vendor HydroBios developed a sampling kit which consists of a non-stick filtering bag and removable cod-ends with a mesh of 50 micron in diagonal dimension. By using various cod-ends large volumes of water can be filtered as cod-ends which became stuck by large organism numbers or a higher sediment load can be replaced. Further an integrated flow meter allows for exact measurements of the filtered water volume[10]. Previous onboard tests have proven that this sampling kit efficiently sampled up to 2,500 litres of water in less than 30 minutes (Fig. 1a). In case of extremely high organisms and/or sediment loads in the water an alternative sampling bag with a larger filtering surface may be used (Fig. 1b).

Fig. 1a. Newly designed ballast water sampling tool. Note water collection buckets (centre) below the nets to avoid water spillage in the ships´ bilge water system. Note further the valve on the right net (left photo, blue circle) which is used to manipulate the water flow to the water sampling net. The removable cod-end is shown on the right photo (Photo, left and centre: Stephan Gollasch, right: Helge Botnen).

Organism survival in the cod-end may be negatively impaired during long sampling times. In case the sampling event takes longer than 30 minutes it is therefore recommended to stop the sampling

[10] To avoid the water supply hoses to disconnect or slip off the flow meter inlet tube ensure that a double set of hose connecting screws are used.

process by closing the valve at the flow meter and to open the cod-end valve to extract the organism into a 1 L sample bottle. This may be done every 15 minutes until the sampling process came to an end.

Fig. 1b. Filtering bag for waters with extremely high sediment and/or organism load (Photo: Helge Botnen).

Multiple sets of this tool will be used to enable replicate samplings (see below sampling at water discharge). In case this sampling equipment becomes stuck by high organisms or sediment loads, water-tight big bags (Fig. 2) will be used to collect the water until the required water volume is met. This water will be filtered through the sampling kit once the clogged cod-end could be cleaned. By doing so the ballast water pumping process does not need to be stopped.

Fig. 2 Water-tight big-bags for water collection (Photo: Helge Botnen).

6.1.3. Organisms below 50 micron and above 10 micron in minimum dimension

Assuming the control and treated tank can be filled simultaneously, for organisms below 50 and above 10 micron in minimum dimension at least 1 litre of water needs to be sampled. This will be done over the entire uptake time (= at least 1 litre of water).

Samples will be collected with a measuring cup multiple times (every few minutes) as a subsample of the water sampled for organisms above 50 micron in minimum dimension, i.e. at the flow meter outlet into the HydroBios sampling nets.

In case the control and treated tank cannot be filled simultaneously one sampling event is needed for each tank, i.e. the above scenario needs to be carried out twice.

6.1.4. Bacteriae

Assuming the control and treated tank can be filled simultaneously, for bacteriae at least 500 millilitres of water needs to be sampled. This will be done over the entire uptake time (= at least 500 millilitres of water).

In case the control and treated tank cannot be filled simultaneously one sampling event is needed for each tank, i.e. the above scenario needs to be carried out twice.

Samples will be collected with a measuring cup multiple times (every few minutes) as a subsample of the water sampled for organisms above 50 micron in minimum dimension, i.e. at the flow meter outlet into the HydroBios sampling nets.

6.2. Samplings at ballast water discharge

The control and treated tank may be emptied consecutively which results in two sampling events during discharge or both tanks may be discharge in parallel. In case the tanks need to be discharged consecutively it is recommended to discharge and sample the treated tank first to avoid contamination with individuals from the likely more organism rich control tank.

6.2.1. Organisms above 50 micron in minimum dimension

For the control tank organisms above 50 micron in minimum dimension at least 1 tonne of water needs to be sampled. This will be done over the entire discharge time (= at least 1 tonne of water).

Samples from the treated tank should be taken in triplicate, i.e. for organisms above 50 micron in minimum dimension at least 3 times 1 tonne of water needs to be sampled. This will be done over the entire discharge time (= at least 3 tonnes of water).

6.2.2. Organisms below 50 micron and above 10 micron in minimum dimension

For the control tank organisms below 50 and above 10 micron in minimum dimension at least 1 litre of water needs to be sampled. This will be done over the entire discharge time (= at least 1 litre of water).

Samples from the treated tank should be taken in triplicate, i.e. for organisms below 50 and above 10 micron in minimum dimension at least 3 times 1 litre of water needs to be sampled. This will be done over the entire discharge time (= at least 3 litres of water).

Samples will be collected with a measuring cup multiple times (every few minutes) as a subsample of the water sampled for organisms above 50 micron in minimum dimension, i.e. at the flow meter outlet into the HydroBios sampling nets.

6.2.3. Bacteriae

For the control tank bacteriae at least 500 millilitres of water need to be sampled. This will be done over the entire discharge time (= at least 500 millilitres of water).

Samples from the treated tank should be taken in triplicate, i.e. for bacteriae at least 3 times 500 millilitres of water need to be sampled. This will be done over the entire discharge time (= at least 1500 millilitres of water).

Samples will be collected with a measuring cup multiple times (every few minutes) as a subsample of the water sampled for organisms above 50 micron in minimum dimension, i.e. at the flow meter outlet into the HydroBios sampling nets.

6.3. Sample number summary

The following table provides a summary of the number of samples and their volumes to be taken during onboard tests for one test cycle (see also Annex 1).

Table 1 Number of samples and their volumes for one test cycle according to G8. In case the treated and control tanks can be filled in parallel one sampling event is sufficient during uptake. It was further assumed that the sampling time stretches over the entire uptake or discharge time of the ballast tanks (see also Annex 1). The numbers referring to paragraphs refer to IMO Guideline G8.

Sample purpose	**Treated tank**		**Control tank**	
	uptake (para 2.2.2.5 and 2.2.2.6)	discharge (para 2.2.2.6.2 and 2.2.2.6)	uptake (para 2.2.2.6.1 and 2.2.2.6.3)	discharge (para 2.2.2.6.1 and 2.2.2.6.3)
Environmental parameters[11]	1 sample	1 sample (recommended, but not required in G8)	1 sample	1 sample (recommended, but not required in G8)
>50 µm[12]	1 x >1000 L (recommended, but not required in G8)	3 x >1000 L	1 x >1000 L	1 x >1000 L
<50 to >10 µm	1 x >1 L (recommended, but not required in G8)	3 x >1 L	1 x >1 L	1 x >1 L
Bacteriae	1 x >500 ml (recommended, but not required in G8)	3 x >500 ml	1 x >500 ml	1 x >500 ml (recommended, but not required in G8)

6.4. Sample processing

The samples should be processed as soon as possible after the sampling event and all attempts should be undertaken to complete the sample analysis of all samples within 6 hours after sampling. The 6 hours time limit refers to relatively short generation times of certain taxa and possible mortality in storage prior to analysis may have an impact even after a few hours.

For all biological samples the likely organism-poor treated water samples should be processed first to avoid organism "contamination" with the likely organism-rich control samples.

6.4.1. Abiotic conditions

The source water for test cycles shall be characterized by measurement of salinity, temperature, particulate organic carbon and total suspended solids[13]. All abiotic parameters should be measured in all samples (but see Table 1).

For salinity measurements a refractometer or calibrated electronic measurement device will be used. A sample volume of less than 1 litre is considered sufficient.

In parallel to salinity measurements, the water temperature should be measured with a standard thermometer. A 10 litre bucket should be filled and the water temperature measured at the sampling point.

[11] Temperature, salinity, total suspended solids and particulate organic carbon, see G8, Annex, Part 2, paragraph 2.2.2.9

[12] There is an inconsistency and unclear wording in G8 regarding the uptake sample of the treated line. G8, Annex, Part 2, paragraph 2.2.2.5 *Valid tests are indicated by uptake water, for both the control tank and ballast water to be treated, with viable organism concentration…* but 2.2.2.6 *Sampling regime* sets only requirements for the discharge of treated water. To be on the save side samples during uptake of the treated water are therefore recommended.

[13] G8, Annex, Part 2, paragraph 2.2.2.9

The particulate organic carbon (POC) and total suspended solids (TSS) analysis will be carried out on land after the voyage.

For TSS, before boarding the ship, fibre filters (e.g. Whitman GF/C) will be dried overnight at 500 °C, weighted and put in numbered and sealed aluminium foil or Petri discs. Onboard, water is filtered and thereafter the filter is rinsed with fresh water (preferably distilled water) and put back in the original Petri disc. The filters are thereafter frozen at ca. – 20 °C (in the ships deep freezer) and should at best be kept frozen until analysis. Preferably 1 litre of water should be vacuum filtered for TSS. The TSS and POC measurements will be performed at NIOZ, Texel, The Netherlands. For sample shipment the sample transfer protocol will be followed (Annex 5).

6.4.2. Organisms minimum dimension measurement

For organisms above 10 micron the minimum dimension measurement should be based upon an investigation of the organism "body", thereby ignoring sizes of thin spines, antenna etc (Fig. 3). In e.g. flat worms or diatoms the minimum dimension should be the smallest part of their "body", i.e. the dimension between the body surfaces when looked at the individual from the side. In ball shaped organisms the minimum dimension should be the spherical diameter.

For analysis of smaller organisms a flow camera of FluidImaging (Fig. 4) may also be used onboard.

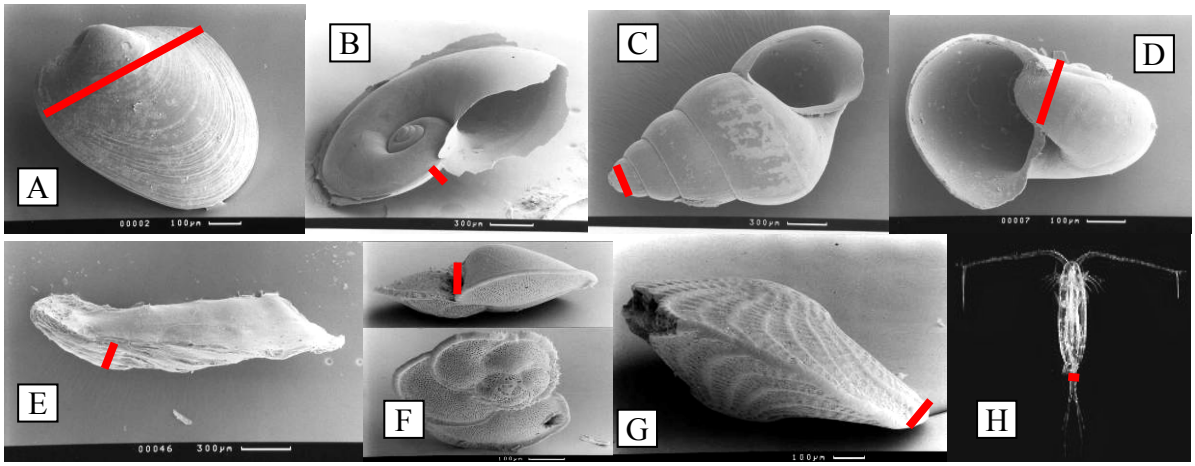

Fig. 3 Minimum dimension measurements (red line) for selected organism types: A = mussel larvae, B to D = gastropod larvae, E = young oyster, F = Foraminiferida (phytoplankton), top shows individual from the top and bottom the same individual from the side, G = Foraminiferida and H = copepod. All organisms shown here are well above 50 micron and organisms shown in A to G were sampled from ballast water during the German shipping study (Photos A - G: Stephan Gollasch, H: www.wikipedia.org).

Fig. 4 The flow camera of FluidImaging may be used to analyse phytoplankton samples onboard (Photo: Stephan Gollasch).

6.4.3. Organism counting

6.4.3.1. Organisms above 50 micron in minimum dimension

Samples will be analysed onboard directly after sampling. A stereo-microscope will be used for counting the larger organisms in the samples (Fig. 5). Illumination is provided from the top or bottom.

Organisms greater than or equal to 50 micrometers in minimum dimension should be counted under magnifications of at least 10 x. For a size measurements a piece of the filtering mesh (50 micron in diagonal dimension) will be put under the stereomicroscope. This transparent mesh is used as a scale and all living organisms above 50 micron in minimum dimension will be counted. The organism numbers will be recorded according to broad taxonomic groups, such as copepods, decapods, polychaetes, bivalves, gastropods, phytoplankton etc. (see Annex 2).

Fig. 5 A stereo-microscope in use for onboard analysis of organisms above 50 micron. A Bogorov counting chamber is shown on the white tray in the foreground (Photo: Stephan Gollasch).

In case samples need to be concentrated a sieve no bigger than 30 micron should be used. The concentrated sample should not be less than 50 millilitres in total volume to avoid negative effects of the densely concentrated organisms.

In organism rich samples (e.g. uptake water, discharge sample of the control tank) the sample may be divided into sub-samples. This may be undertaken by proper mixing of the sample (stir well until organisms are equally distributed in the sample) and extracting when practicable approximately 10 millilitres with a pipette[14] for analysis. Organism poor samples (e.g. the samples taken from the treated tank upon discharge) should be analysed completely when practicable.

When using Petri discs counting may not be accurate as the ship movement induces water movements in the Petri disc. As a result organisms may be counted twice and some may be missed out from counting. To avoid this, a Bogorov counting chamber may be used. During minimal ship movements, this chamber proved to be efficient during onboard trials. However, with increasing ship movements the Bogorov chamber looses its advantage. HydroBios therefore designed three new counting chambers which may be used with increasing ship movements. Using these gives a

[14] A new pipette will be used for each sample to avoid organism contamination.

much greater accuracy as here the size of the water canal width corresponds to the stereomicroscope observation field of view thereby reducing the risk to overlook organisms (Fig. 6).

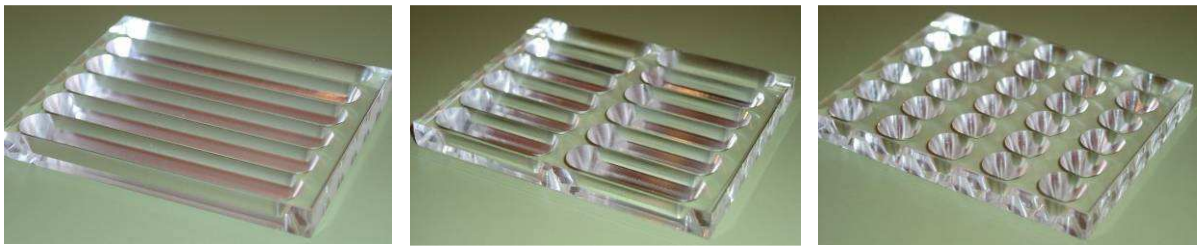

Fig. 6 Newly designed counting chambers of organisms above 50 micron in minimum dimension. Photos courtesy of www.hydrobios.de.

6.4.3.2. Organisms below 50 and above 10 micron in minimum dimension

For analysis of smaller organisms a flow camera of FluidImaging (Fig. 4) may be used onboard. This camera will automatically count objects per size class and is also able to assess organism viability for phytoplankton (see below).

Alternatively, filter at least 1 L of water per sample via a 5 µm mesh and transfer to sample jar. One set of samples will be preserved using Lugol Solution and another set be kept alive in a fridge. Both sets will later be analysed in a land-based laboratory (at NIOZ, Texel, The Netherlands). In case samples will be sent to a land-based laboratory the sample transfer protocol will be followed (Annex 5).

Colony forming species - A question arose in which size category a colony falls when the single cell is below 50 micrometres but the colony is above 50 micrometres. A team of experts, i.e. the ICES/IOC/IMO Working Group on Ballast and Other Ship Vectors, believes that in those cases the individual specimen size should be measured. This group finding is based upon the D-2 standard as it refers to organisms and not to colonies. Further, viability assessments (see below) should address the smallest unit able to reproduce which is the individual and not the colony. Based on this conclusion the size of the individuals should be measured and not the colony.

6.4.3.3. Bacteriae

Except for *Vibrio cholerae* bacteriae will be cultured onboard using selective media (see below). Cholerae bacteriae will be analysed in a certified laboratory on land. The most appropriate lab will be selected according to their availability. Two laboratories have been identified to undertake such sample analysis, i.e. IBEN in Bremerhaven, Germany and Analycen in Moss, Norway.

6.5. Viability assessment

6.5.1. Organisms above 50 micron in minimum dimension

The organism viability may be assessed by visual inspection, i.e. all individuals should be inspected for movement or organ activity. The life/dead judgement should be undertaken by exposure of the organisms to light (under the stereomicroscope) and in addition non-moving organisms should be poked with a needle to initiate movement. Intact and moving organisms should be considered as living, organ activity of intact organisms gives an additional indication of viability. This assessment should be undertaken onboard and completed for all samples no later than 6 hours after sampling.

6.5.2. Organisms below 50 micron and above 10 micron in minimum dimension

Stains together with epifluorescence microscopy or flow cytometry will be used to proof viability of phytoplankton organisms (Fig. 4). In general these tools are applied to distinguish living

phytoplankton from other detrital material and zooplankton based on the presence of auto chlorophyll fluorescence of phytoplankton.

To proof viability of phytoplankton the Pulse-Amplitude Modulated fluorometer method will, whenever possible, be used onboard. This is especially valid on longer voyages, i.e. where the sampling and sample analysis is more than 10 days apart. In these cases the phytoplankton survival in the samples stored in the fridge onboard may be impaired which consequently has a negative influence on sample processing. As a result false positives of treated water samples may occur as the phytoplankton organisms may have died in storage. Further, the minimum intake concentration of living organisms may not be met for the same reason.

PAM fluorometry
The photochemical efficiency of photosystem II (an indicator of the 'health' condition of the cell) of phytoplankton can be assessed using the Pulse-Amplitude Modulated fluorometer (PAM-fluorometry; Schreiber et al 1993[15], Fig. 7). This simple parameter gives a qualitative indication of the photosynthetic activity of the phytoplankton community.

The PAM-fluorometer will be calibrated onboard with destilled water and the result taken as the "zero sample". After calibration ca. 3 ml of the filtered sample water are filled into a glass cuvette and are analysed using the PAM-fluorometer in an automated measurement. As a result the viability of the phytoplankton cells is measured. This experiment will be repeated at NIOZ with the unpreserved samples. The Lugol preserved samples will only be analysed in the NIOZ laboratories for the number of cells in the sample should the unpreserved samples show contrary results. Consequently, the onboard viability test in combination with the land-based sample analysis provide the necessary results of viable cells per water volume. The onboard measurement should be undertaken and completed for all samples no later than 6 hours after sampling.

Fig. 7 PAM fluorometry; a fast method to determine (bulk) phytoplankton biomass and the physiological condition of the photosynthetic apparatus of the cells.

Organisms should be counted by using magnifications of at least 100 x of an inverted microscope. Stains may also be used to assess organism viability. In phytoplankton species an inspection of the photopigments may reveal additional information to assess viability.

[15] Schreiber U, Neubauer C, Schliwa U (1993) PAM fluorometer based on medium-frequency pulsed Xe-flash measuring light: A highly sensitive new tool in basic and applied photosynthesis. Photosynth. Res. 36:65 - 72

6.5.3. Bacteriae

Selective growth media, an immunoassay or quantitative PCR (Polymerase chain reaction) should be used for the three categories of organisms mentioned in G8 i.e. toxigenic *Vibrio cholerae* (serotypes O1 and O139), *Escherichia coli* and intestinal *Enterococci.*

Where appropriate a dilution series will be applied to ensure proper counting of bacteriae. By doing so bacteriae counting is enabled even in the unlikely case of high concentrations. The different dilution cultures will be used to back-calculate the bacteriae densities in the original sample. Where appropriate, for each bacterial sample at least three culture trials should be started in parallel.

To avoid contamination use the maximum water temperature in cabin sink to clean gear when starting to process another type of bacteriae. It is further recommended to clean the desk and other working items with Ethanol prior sample processing.

6.5.3.1. Vibrio cholerae (serotypes O1 and O139)

Onboard analysis is not permitted due to safety concerns onboard. To analyse for the abundances of O1 and O139, samples will be stored for later analysis, e.g. by a monoclonal antibody tagged with fluorescein isothiocyanate (FITC) immunoassay for specific disease causing strains. Alternatively quantitative PCR (Polymerase chain reaction) may be used, i.e. "copy" DNA, proof presence, multiply with dilution factor to back-calculate original numbers.

Samples will be sent to a land-based laboratory and the sample transfer protocol will be followed (Annex 5).

Onboard a Millipore filter set will be used to filter 100 ml of undiluted sample through a Whatman filter (0,45 µm pore size) (Fig. 8). Before doing so, clean filter stand with Ethanol and flame it (Fig. 9).

Put filter in container, add a few millilitres of filtered water to avoid the filter to dry (Fig. 10) and keep in incubator (Fig. 11) at 37 °C for later analysis on land.

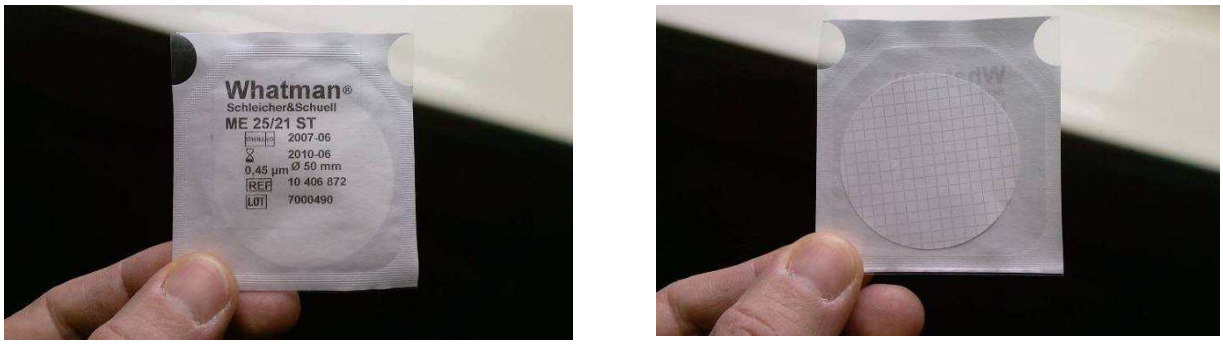

Fig. 8. Whatman filters (0,45 µm pore size) (Photo: Stephan Gollasch).


Fig. 9 Cleaning of Millipore stand (Photo: Stephan Gollasch).


Fig. 10 Whatman filter with *V. cholerae* sample (Photo: Stephan Gollasch).


Fig. 11 Culture chambers for bacteriae storage and processing for onboard use (Photo: Stephan Gollasch).

6.5.3.2. Escherichia coli

It is recommended to follow the ISO standard 9308-1:2000 to analyse the samples for *E. coli* concentrations. A selective medium for this bacterium and the analysis is relatively easy, i.e. can be carried out onboard. GoConsult is prepared to work according to ISO 9308-1:2000 and a bacteriae culture chamber is available.

Use Millipore filter set to filter, when appropriate, 1 ml, 10 ml and 100 ml of undiluted sample through Whatman filter (0,45 µm pore size) (Fig. 8). Before doing so, clean filter stand with Ethanol and flame it (Fig. 9).

Moisture the Petri disc with selective medium (Dr. Möller & Schmelz Lactose-TTC-Tergitol NPS (Order No. 1092)) with 3 ml of aqua dest. Add Whatman filters to Petri disc with medium. Incubate 21 +/- 3 hours at 36 +/- 2 °C. *E. coli* forms flat yellow colonies with yellow halo visible from top and bottom view of Petri disc (Fig. 12). Lactose negative bacteriae are either inhibited or develop small reddish colonies.

However, this method was developed to check for lower concentrations of *E. coli*. For higher bacteriae densities this method is not suitable and here ISO 9308-3 is recommended. However, ISO 9308-3 cannot be carried out onboard ships as microtiter plates cannot be sealed which may result in water spillage due to heavy seas or when the samples need to be carried from the ship to the port for an extended culture period. A second method in accordance with ISO 9308-3 was developed by idexx. This requires larger incubators to store the idexx culture plates and also additional gear, i.e. a sealer to close the culture plates. This additional gear complicates the logistics especially as all material needs to be shipped by air travel to and from the vessel. Further, earlier studies have shown that the bacteriae density in sea water is very low. Consequently, due to low bacteriae concentrations in sea water and also logistical problem the suitability of ISO 9308-1:2000 is indicated. To ensure that even in exceptionally higher bacteriae concentrations samples can be analysed a dilution series will be applied.

Please note also that the application of ISO 9308-1:2000 for the onboard analysis of *E. coli* in tests of ballast water treatment systems was previously accepted by the Bundesamt für Seeschifffahrt und Hydrographie, Hamburg, Germany and also Det Norske Veritas, Oslo, Norway.

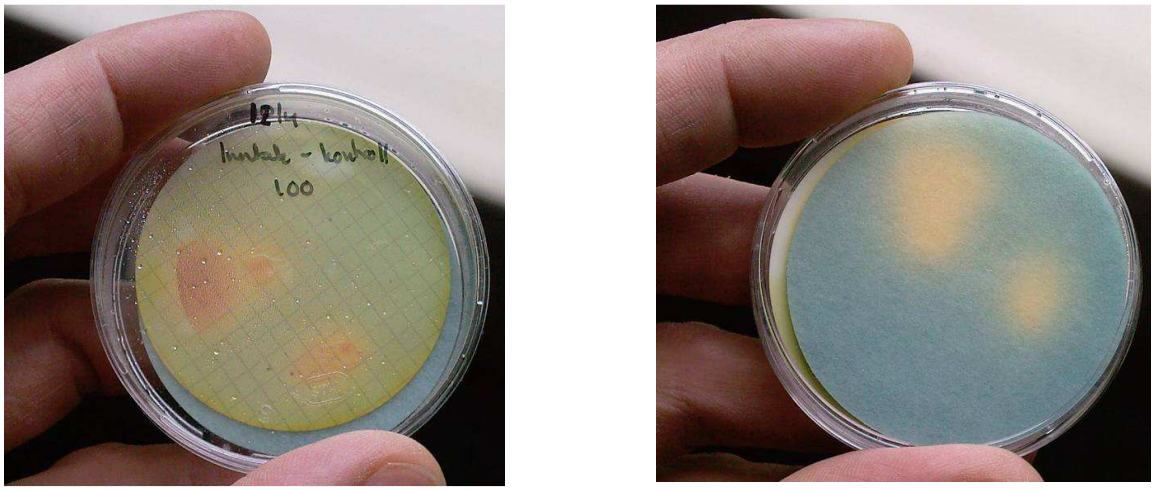

Fig 12. Positive *E. coli* colonies on selective medium. Left = top, right = bottom view of Petri disc (Photo: Stephan Gollasch).

As a second step to proof positive *E. coli* colonies on the selective medium, transfer positive colonies to Tryptophane medium and incubate for 1 day at 44 °C. Positive *E. coli* are indicated by a colour change of the Tryptophane medium from yellow to red/purple when adding two drops of Kovacs solution: CH_3-$(CH_2)_3$-OH (Fig. 13, see test tube behind white Kovac solution bottle).

Fig. 13 Tryptophane medium incubated to proof *E. coli*. Kovacs solution in the front (Photo: Stephan Gollasch).

6.5.3.3. Intestinal Enterococci

It is recommended to follow the ISO standard 7899-2:2000 to analyse the samples for *Enterococci* concentrations. A selective medium for this bacterium and the analysis is relatively easy, i.e. can be carried out onboard. GoConsult is prepared to work according to ISO 7899-2:2000 and a bacteriae culture chamber is available.

Use Milipore filter set to filter, when appropriate, 1 ml, 10 ml and 100 ml of undiluted sample through Whatman filter (0,45 µm pore size) (Fig. 8). Before doing so, clean filter stand with Ethanol and flame it (Fig. 9).

Moisture the Petri disc with selective medium (Dr. Möller & Schmelz *Enterococcus* selective NPS medium (Order No. 1091)) with 3 ml of aqua dest. Add Whatman filters to Petri disc with medium. Incubate in minimum 24 and at best 48 hours at 36 +/- 2 °C (Fig. 11). *Enterococci* form small pink or dark red colonies.

7. Cleaning

7.1. Treatment system

The ballast water treatment system needs to be clean, i.e. shall not contain living organisms prior each test run. This requires proper flushing and e.g. filter cleaning where appropriate.

It should be noted that organism can survive in the ballast water pipe work of the vessel. When working on ballast water discharge samples it is therefore recommend to work from organism poor to organism rich samples to avoid organism "contamination" between such samples. As a consequence the treated tank should be emptied first and before the sampling starts flushing of the ships ballast water pipe should be undertaken to ensure that only treated water is sampled and not untreated water from previous ballast water operations after the treated tank was filled.

7.2. Sampling equipment

After each sampling trial is completed, all sampling equipment and sample processing devices should be cleaned by using freshwater. All gear should also be inspected before any new samples are taken to prevent contamination with organisms from the previous sampling.

7.3. Ballast tank

It is also recommended that the selected ballast tanks should be cleaned before commencing a test cycle. This may be achieved by multiple tank washings using e.g. the empty/refill method. Otherwise, organisms remaining in the tank from previous ballast water operations (incl. earlier treatment system tests) could interfere with the results.

8. Reporting and communication

The appointed sampling team leader (see Annex 3) will be responsible to record the data during sampling events in the engine room. The essentials to document during uptake and discharge samplings at the sampling point are given in table format in Annex 7. These data should be transferred to the sample analysis sheet for organisms above 50 micron in minimum dimension (Annex 2) and also to complete the Test Cycle Report (Annex 6).

Items needed at the sampling point for uptake and discharge sampling events are given in Annex 9. Steps to set up the sampling gear are outlined in Annex 11.

For sample labelling use the labelling suggestions given in Annex 10.

The appointed sampling team leader will also be responsible to prepare test cycle result reports for each individual test cycle undertaken. These reports may follow the structure as described in Annex 6.

Ensure that all samples for later land-based analysis are taken from the vessel (Annex 12).

As it is sometimes essential to arrange a meeting with all sampling team members at short notice a "**sampling team locator"** may be printed and hang on the cabin door of each sampling team member (Annex 8).

Before leaving the vessel all documents prepared during the voyage should also be copied to a memory stick. It is further advisable that both sampling team members of GoConsult carry a copy of all such documents. Key documents, such as test cycle result reports, should also (when possible) be printed and handed over to all sampling team members.

Other means of communication and responsibilities are outlined in the Quality Assurance Project Plan (QAPP) for Shipboard Tests of Ballast Water Management Systems (Annex 3).

9. Heavy seas

Safety first! The sampling team should always use "one hand for themselves and the other for the vessel", i.e. when carrying material to the sampling point of back to the cabin, carry only one bucket or bag at a time and hold to the hand rail, especially on stairs.

In case of heavy seas all fragile items must be placed on the floor, including

- incubators
- stereo microscope

- flow meters
- computer

When using the material above, even at calm seas always use an anti-slip matt on the working desk.

To avoid sample jars (concentrated zoo- and phytoplankton samples) and preservatives to flip over, put these jars in empty coffee mugs from the ship with some Kleenex. Mugs less likely flip over as they have a better stand.

Annex 1 Number of samples

Number of samples according to the IMO *Guidelines for Approval of Ballast Water Management Systems* (G8) with reference to the relevant paragraphs in the guideline. Samples highlighted in yellow are not required according to G8, but it is recommended to take those samples in the interest to better proof the treatment systems performance. **A** = number of samples required when samplings are undertaken at the beginning, middle and end of an uptake and discharge event, **B** = number of samples to take when sampling over the entire uptake and discharge time (preferred option).

A	**Treated water**			**Control**		**Total**
	uptake before treatment	**immediately after treatment**	**upon discharge (para 2.2.2.6.2)**	**uptake (para 2.2.2.6.1)**	**upon discharge (para 2.2.2.6.1)**	
Test cycle 1						
> 50 µm	3	1	9	3	3	19
10 - 50 µm	3	1	9	3	3	19
Bacteria	3	1	9	3	3	19
Test cycle 2						
> 50 µm	3	1	9	3	3	19
10 - 50 µm	3	1	9	3	3	19
Bacteria	3	1	9	3	3	19
Test cycle 3						
> 50 µm	3	1	9	3	3	19
10 - 50 µm	3	1	9	3	3	19
Bacteria	3	1	9	3	3	19

B	**Treated water**			**Control**		**Total**
	uptake before treatment	**immediately after treatment**	**upon discharge (para 2.2.2.6.2)**	**uptake (para 2.2.2.6.1)**	**upon discharge (para 2.2.2.6.1)**	
Test cycle 1						
> 50 µm	1	1	3	1	1	7
10 - 50 µm	1	1	3	1	1	7
Bacteria	1	1	3	1	1	7
Test cycle 2						
> 50 µm	1	1	3	1	1	7
10 - 50 µm	1	1	3	1	1	7
Bacteria	1	1	3	1	1	7
Test cycle 3						
> 50 µm	1	1	3	1	1	7
10 - 50 µm	1	1	3	1	1	7
Bacteria	1	1	3	1	1	7

Comments	
For storage no minimum time required per cycle, all 3 cycles over at least 6 months (para 2.2.2.7)	
Valid ship-board tests (para 2.2.2.5)	
	min. intake concentration >50 = 10 x D-2 = >90 ind./tonne
	min. intake concentration <50 and >10 = 10 x D-2 = >90 ind./ml = 90.000 pro L
	min. intake concentration heterotrophic bacteriae - not required
	control tank at discharge at least D-2.1
Sample volumes (para 2.2.2.6.3)	
intake water	orgs >50 volume at least 1 to
	<50 - >10 volume at least 1 litre
	bacteriae >500 ml
discharge water	orgs >50 volume at least 1 to
	<50 - >10 volume at least 1 litre
	bacteriae >500 ml

Annex 2 Sampling event details and zooplankton analysis sheet.

Sampling day			Temperature °C		Salinity PSU			
Location			TSS filter number					
Sample type			Comment					
Sampling time	start end		Largest organism					
Sample volume	unconcentrated							
	concentrated							
	analysed	2 ml x						
	Copepoda	Rotatoria	Cladocera	Mollusc larvae	"Worms"			
living[16]								
total:								
(dead)								

Total number of living individuals in 1000 L:

Sampling day			Temperature °C		Salinity PSU			
Location			TSS filter number					
Sample type			Comment					
Sampling time	start end		Largest organism					
Sample volume	unconcentrated							
	concentrated							
	analysed	2 ml x						
	Copepoda	Rotatoria	Cladocera	Mollusc larvae	"Worms"			
living								
total:								
(dead)								

Total number of living individuals in 1000 L:

Date

Signature

[16] Each line stands for one sub-sample analysis, i.e. one line per HydroBios counting plate analysed. Record organism numbers of all six plate channels separately.

Annex 3 QAPP

Quality Assurance Project Plan (QAPP) for Shipboard Tests of Ballast Water Management Systems

Introduction

In this QAPP, vendor means the Aquaworx ATC GmbH, Munich, Germany (hereafter vendor). The sampling crew will consist of two GoConsult experts (see Annex 4). It is also expected that at least one member of the vendor will join the sampling team to run the treatment system which should only be done in close consultation with the vessel crew.

Personnel and responsibilities

The work load during sampling and sample processing requires a sampling crew of at least two trained and experienced members, and it is recommended that each have their own specialities on sample processing. It is therefore recommended that the sampling crew consist of one (zoo-) plankton specialist and one microbiologist. Each sampling crew member is responsible to bring the equipment needed for the onboard analysis (Annex 9).

One sampling team member may be replaced by a generalist, particularly if some of the samples will be processed later in a land-based laboratory.

All sampling crew members planning to send samples to land-based laboratories should follow the Sample Transfer Document (see Annex 5).

One of the GoConsultians will be appointed as sampling crew leader on each voyage.

During each test cycle the sampling crew will make sure that the sampling equipment is cleaned prior each sampling event, that the equipment is correctly installed, that the samples are taken from correct sampling points, that the necessary number and volume of samples are correctly taken and that sample handling and sample processing is correctly performed.

Responsibilities of the sampling crew leader

- Communication concerning the organisational aspects of the test between sampling crew (see sampling team locator, Annex 8) and the crew of the treatment system vendor and ship crew
- In close consultation with the crew of the treatment system vendor/ship crew the sampling crew leader will decide
    - when to start ballast water uptake
    - when to start the ballast water uptake sampling event
    - when to finish the sampling
    - when to start the ballast water discharge
    - when to start the ballast water sampling during discharge
    - when to finish the ballast water discharge sampling event
- Responsible for the set-up of sampling gear at the sampling point (Annex 11)
- Responsible for shipment of samples, if necessary (see Sample transfer protocol, Annex 5 and Sample transport reminder, Annex 12)
- Responsible for documentation (see Sampling event details, documentation at the sampling point, Annex 7; Sample labelling, Annex 10) and reporting of the results (see Test cycle report, Annex 6)

The leader of the sampling crew can transfer responsibilities or duties to other sampling crew members provided such transfers are properly documented.

Responsibility of specialist for organisms larger than 50 µm

- Prepare all equipment for sampling (see list of items needed at the sampling point, Annex 9)
- Prepare all equipment for sample processing
- Process and analyse all samples onboard
- Reporting of results to the sampling crew leader

Responsibility of specialist for organisms between 10- 50 µm

- Prepare all equipment for sampling (see list of items needed at the sampling point, Annex 9)
- Prepare all equipment for sample processing
- Process all samples
- Reporting of results to the sampling crew leader

Responsibility of microbiological specialist (bacteria)

- Prepare all equipment for bacteria sampling (see list of items needed at the sampling point, Annex 9)
- Process and analyse all samples onboard (*E. coli, Enterococci*)
- Prepare all bacteria samples for later land-based analysis (*Vibrio cholerae*)
- Reporting of results to the sampling crew leader

Responsibility of generalist

- Support the installation of sampling equipment
- Measure temperature
- Measure salinity
- Collect sample for TSS/POC
- Document other relevant data, such as start and end time of the sampling event, lat/lon data, water depth, distance to nearest land for the starting and endpoint of the sampling event (interview responsible officers at the wheel house)
- Optional photo documentation of the sampling event
- Reporting of data to the sampling crew leader

Reporting of test results

After each test cycle a test cycle report will be prepared by the sampling crew leader (see Annex 2).

Equipment, check lists, for one test[17]

Bacteria sampling and processing

- At least six sterile bottles to store *Vibrio cholerae* samples
- Two incubators with adjustable temperature and thermometers
- *Enterococci* kit (media)
- *E. coli* kit (media)
- Inoculating loops
- Distilled water
- Alcohol (at least 70 % Ethanol or similar) for disinfection of equipment
- Forceps
- Single use latex gloves
- Cylinder for water volume measurement

[17] Items needed for uptake and discharge samplings at the sampling point see Annex 9.

- 1000-5000 ml Eppendorf pipette with tips, or 1 and 5 ml syringes
- Plastic bags to seal Petri discs
- Whatman or Millipore filters 0.45 µm
- Culture media (Dr. Möller & Schmelz, Tryptophane)
- Kovacs solution
- Tryptophane medium in test tubes
- Test tube stand
- Millipore stand, with hoses (and vacuum flask/pump)
- Filtration funnels, sterile
- Burner to flame Millipore stand (as replacement bring a conventional lighter or matches)
- Paper towels
- Material to label samples (see Annex 10)
- Copies of Sample Transfer Protocol for Cholera-samples (Annex 5)

Smaller plankton (10-50 µm)

- At least six 50 ml bottles
- Optional: Flow camera and relevant gear
- Small plastic bags
- Lugol
- Pipette for adding Lugol to samples
- Small sieves with removable 5 µm plankton mesh (one for each sample)
- Measuring cups 0,5, 1 and 2 L
- Lugol solution and pipette
- Material to label samples (see Annex 10)
- Small plastic bags (2 L) to store samples
- Transparent tape to secure label on samples
- Cooling containers with cooling units
- Robust tape to seal cooling containers
- Copies of Sample Transfer Protocol (Annex 5)

Larger plankton (larger than 50 µm) sampling and processing

- Four HydroBios plankton nets
- Four flow meters
- At least five removable cod-ends
- Replacement filtering mesh (one for each cod-end)
- At least 4 buckets, preferably with lids
- Wash bottles
- Small sieves with removable 30 µm plankton mesh (one for each sample)
- Forceps
- Needles for poking (life/dead judgement)
- Funnel
- Pipettes
- Small measuring cup (100 ml) for concentrated sample
- Stereo microscope
- Replacement light bulb
- Counting chambers (one canal per row, two canals per row and with holes)
- Paper towels
- Copies of Test Cycle Report (Annex 6)

TSS, POC, temperature and salinity

- Filtering stand for filter (diameter 47 mm)
- Pre-weighted GF/C filters
- Wash bottle
- Forceps
- Aluminium foil or small plastic bags
- Volume measurer
- Filtration funnels, not sterile
- Small plastic bags (2 L) to store samples
- Thermometer
- Salinitymeter
- Material to label samples (see Annex 10)
- Copies of Sample Transfer Protocol (Annex 5)
- (Optional: pH measuring strips)

Additional requirements for all samplings

- The sampling points must be correctly installed by the vendor
- All sampling points should be of identical design
- Hoses for water transfer from sampling point to position for sample collection
- Where appropriate, water tanks and a draining system at the position of the sample collection point
- Watch
- Paper and writing utensils
- Laptop computer
- Memory stick
- Photo camera
- Waterproof marker
- Suitable boxes, sampling bottles, buckets for transportation of sampling equipment, including Styrofoam boxes with cooling units

Work clothes

- Light boiler suit
- Hearing protection
- Suitable footwear, e.g. steel-cap rubber boots
- Gloves
- (Optional: hard hat)

Transfer of samples to any land based laboratory

In case of sample transfer, a transfer document must be issued (Annex 5). The document must state all parties being involved in the transfer, type of sample (water, filter etc.), number of samples, type of analysis (phytoplankton, TSS, Cholera etc.), date of transfer and signature of all party representatives. The sample recipient laboratory should copy the signed sample transfer document and make this available to the sampling crew. The transfer document should be kept in the archives of the sampling crew.

Please note also the sample transfer reminder (Annex 12).

Annex 4 Brief description of sampling team expertise

To avoid unavailability due to an overlap of parallel sampling events GoConsult has two sampling teams available. One consists of Stephan Gollasch and Matej David, the other of Dan Minchin and Peter Hahn. A three or four personnel sampling team can also be arranged for.

Stephan Gollasch, marine biologist (PhD), born in Hamburg, Germany in 1962. Current position: senior scientist at GoConsult, an independent consultancy company based in Hamburg, Germany (www.gollaschconsulting.de).
Since 2002 Gollasch is involved in various projects and programmes dealing with ballast water treatment systems, including land-based and ship-board efficacy tests. Other fields of expertise include risk assessment and the development of ballast water management scenarios. Since 1994 Gollasch is member of the German Delegation at IMO MEPC and represents his country in the Ballast Water Working Group. From the beginning he is member of the ICES/IOC/IMO Working Group on Ballast and Other Ship Vectors and was chairman of the group from 2000 to 2006. Gollasch sampled the ballast water of more than 200 vessels and spent almost 200 days at sea during various tests of ballast water treatment systems.

Matej David (PhD) was born in Koper, Slovenia, in 1967. He finished navigation studies (Maritime Transport Engineer), sailed on merchant vessels and holds the License of Competence as Ship's Mate (officer). Since 2001 he is involved in ballast water researches. In 2002 he started as member of the Slovenian Delegation at IMO MEPC and also represents his country in the Ballast Water Working Group. In 2003 he was the leading scientist in a ballast water sampling study undertaken in the Port of Koper, Slovenia. He developed new ballast water sampling gear which was patented in 2003. In 2007 he got his doctoral degree in the field of maritime transport with a focus on ballast water management decision support aspects. In the Port of Hamburg, Germany, he was familiarized with the operation and sampling of a ballast water treatment system on a land-based test site (i.e. performance tests) and he was involved in more than 10 voyages to test ballast water treatment systems performance according to the IMO Guideline G8 including several voyages where he was the appointed sampling team leader..

Jonathan **Dan Minchin** (PhD), born in Galway, Ireland in 1946. He has a broad knowledge of marine biota. He is a scientific diver and holds safety at sea certificates. He has specialised in aquatic alien species and has a background in aquaculture, fisheries and environmental management. He is a senior investigator on hull fouling and ballast water alien transmissions. He has been senior scientist on zooplankton research cruises. He has been a member of the ICES/IOC/IMO Working Group on Ballast and Other Ship Vectors representing Ireland. In the Port of Klaipeda, Lithuania he was familiarized with the operation and sampling for efficacy tests of a ballast water treatment system onboard a commercial vessel. Following additional training sessions at GoConsult he was involved in several voyages to test ballast water treatment systems performance according to the IMO Guideline G8.

Peter Hahn, assistant at GoConsult, born in Stuttgart, Germany 1953. Hahn was involved in planning and organizing onboard sampling events and further ensures appropriate sample transfer into land-based analytical laboratories. He works for GoConsult since 2006 and was involved in various onboard tests of two ballast treatment systems.

Annex 5 Sample Transfer Protocol

Sample Transfer Protocol

Project details: e.g. AquaTriComb^{TM}, onboard test 1, dd.mm.yyyy to dd.mm.yyyy

The responsibility of these samples is transferred to the sample recipient by the signature of the recipient below.

Owner of sample(s) and results: Aquaworx ATC GmbH, München, Germany

Sample recipient: details of analytical laboratory

Comment:
e.g. Please check for toxicogenic *Vibrio cholerae* (O1 and O139) and document density of colony forming unit (cfu) per 100 millilitres.
e.g. Please check for viable organisms below 50 and above 10 micron in minimum dimension and report densities of such individuals per water volume. Please measure volume of water in sampling jar.

Number of samples:

Sample labelling

Sample type	**Sample label**	**Sample type**	**Sample label**
…			

Date and signature of sample owner:

Date and signature of sample recipient:

Date and signature of sample recipient:

Date and signature of sample recipient:

Date and signature of sample recipient:

Annex 6 Test Cycle Report

Test Cycle Report

Treatment system: AquaTriComb™
Ship name: Cellus
Date and time for ballast water uptake: dd.mm.20xx, 20:03 - 20:17.

- Position of ship during beginning of ballast water uptake: region, ca. 28.5 N, 33.8 E.
- Distance travelled during uptake was ca. 14 nm.
- Water depth ca. 60 m.
- Distance to nearest main land ca. 8 nm.

Date and time for ballast water discharge: dd.mm.20xx, 20:40 - 21:42.

- Position of ship during beginning of ballast water discharge: ca. 29.1 N, 34.4 E.

Holding time of ballast water between uptake and discharge ca. 20 hours.
Weather conditions during the test: good, very little ship movements.

Water quality and number of organisms in uptake and discharge water

Parameter	Unit	Uptake water[18]			Discharge water						
		control	before treatment	IMO	control	IMO[19]	treated			aver.	IMO
							# 1	# 2	# 3	#1-#3	
Temperature	°C			-		-					-
Salinity	PSU			-		-					-
POC *	mg/l			-		-					-
TSS *	mg/l			-		-					-
Sample vol. >50 µm	Litres			>1000		>1000					>1000
Organisms >50µm	org./1m³			>90[20]		>10					<10
Sample vol. 50-10 µm	Litres			>1		>1					>1
Organisms 10-50µm*	org./1ml			>90[21]		>10					<10
Sample vol. bacteria	Litres			>0,5		-					>0,5
Escherichia coli	cfu/100ml			-		-					<250
Intestinal *Enterococci*	cfu/100ml			-		-					<100
*Vibrio cholerae***	cfu/100ml			-		-					<1

* Analyzed at NIOZ, Texel, The Netherlands. ** Analysed at IBEN, Bremerhaven, Germany.

Remarks: describe problems during sampling and sample analysis

Conclusions: test validity, compliance with D-2

Date and signature of the sampling crew leader

dd.mm.20xx S. Gollasch

><((((°>
GoConsult
Hamburg

[18] IMO does not require to sample the treated line during uptake (G8, Annex, Part 2, paragraph 2.2.2.6.2). However, the wording is unclear and consequently such samplings are recommended (see sampling protocol).
[19] IMO does not require the bacteriological analysis of the control discharge water (G8, Annex, Part 2, paragraph 2.2.2.5). Any weakness here will not influence the test validity.
[20] current version of G8, Annex, Part 2, paragraph 2.2.2.5 (see sampling protocol)
[21] current version of G8, Annex, Part 2, paragraph 2.2.2.5 (see sampling protocol)

Annex 7 Sampling event details, documentation at the sampling point

The following data need to be recorded during the ballast water sampling at the sampling point, e.g. in the engine room. The placeholders may be deleted and the tables be printed for onboard use.

Ballast water **uptake**

Date	dd.mm.yy	
Sampling duration	start: hh:mm, end hh:mm	
Ship position	e.g. off Pacific Mexico	
Distance to nearest land	[nm]	
Water depth	from xx m to yy m	
Comment		
Sample type	Temperature	Water volume filtered
• control		
• treated		

Ballast water **discharge**
(R1 - R3 are the three replicate samplings required at the discharge of the treated water).

Date	dd.mm.yy	
Sampling duration	start: hh:mm, end hh:mm	
Ship position	e.g. off Pacific Mexico	
Comment		
Sample type	Temperature	Water volume filtered
• control		
• treated, R1		
• treated, R2		
• treated, R3		

Annex 8 Optional: Sampling team locator

As it is sometimes essential to arrange a meeting with all sampling team members at short notice the following may be printed and hang on the outside of the cabin door of each sampling team member. A magnet may be used to indicate where the team members are. This should be updated whenever a team member leaves the cabin.

><((((°>

GoConsult

Hamburg

Name of sampling team member

Please find me:

- here in my cabin, please come in
- here in my cabin, **please do only disturb in urgent cases!**
- in the cabin of the other GoConsult sampling team member
- in the cabin of the ballast water treatment system personnel
- in the engine room
- in a meeting at the ships office
- in a meeting with the sampling responsible ships crew
- in the laboratory
- on the wheel house
- out for a meal
- on deck
- at the gym

Annex 9 Items needed for uptake and discharge samplings at the sampling point

Items to be brought to the sampling point:

Item	Number	
	uptake*	**discharge****
Writing utensils (pencil, paper, waterproof pen)	1 set	1 set
Tape (to avoid net damage/cuts from metal screws used to fit hose to flow meter inlet tube)	1	1
Bucket with lid	2	4
Thermometers	2	4
Bottles with wide opening ("continuous" drip sample)	2	4
Wash bottles	2	4
HydroBios plankton nets	2	4
Rope to hang plankton nets	2	4
HydroBios cod-ends (one as replacement)	3	5
HydroBios filtering socks (as replacement)	3	5
1 L sample bottles with lids to store organism extracted from cod-end during longer sampling events	2	4
HydroBios inline flow meter[22]	2	4
Replacement sealing rings for flow meters	4	8
Optional: big bags with water tight inlay to be used in case cod-ends become completely clogged	2	2

* During uptake one sample is taken from the control line and another from the treated line.
** During discharge one sample is taken from the control line and three from the treated line (as replicates).

It is further recommended to bring hearing protection, a towel and some drinking water for longer sampling events.

[22] To avoid the water supply hoses to disconnect to slip off the flow meter inlet tube ensure that a double set of hose connecting screws are used.

Annex 10 Sample labelling

Except samples for organisms above 50 micron in minimum dimension, which will be analysed directly after sampling, each sample needs to be clearly labelled. The following is a labelling suggestion. The placeholders may be deleted and the tables be printed for onboard use.

TSS/POC
Write directly on Petri disc with waterproof pen. Secure both Petri disc plates with transparent tape. Record Petri disc number on *Sample event details and zooplankton analysis sheet* (Annex 2).

Uptake sampling

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Control

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated

Discharge sampling

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Control

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 1

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 2

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 3

Organisms less than 50 and bigger than 10 micron in minimum dimension

Use self-glue paper stickers, write with pencil. Place sticker on the side of the sampling bottle and secure with transparent tape. In case the PAM-fluorometry is used onboard no living samples need to be stored.

Uptake sampling

Living Lugol preserved

dd.mm.yy	dd.mm.yy
Uptake	Uptake
Volume filtered	Volume filtered
Region (e.g. off Pacific Mexico)	Region (e.g. off Pacific Mexico)
Control	**Control**
Measure volume!	Measure volume!

dd.mm.yy	dd.mm.yy
Uptake	Uptake
Volume filtered	Volume filtered
Region (e.g. off Pacific Mexico)	Region (e.g. off Pacific Mexico)
Treated	**Treated**
Measure volume!	Measure volume!

Discharge sampling

Living Lugol preserved

dd.mm.yy	dd.mm.yy
Uptake	Uptake
Volume filtered	Volume filtered
Region (e.g. off Pacific Mexico)	Region (e.g. off Pacific Mexico)
Control	**Control**
Measure volume!	Measure volume!

dd.mm.yy	dd.mm.yy
Uptake	Uptake
Volume filtered	Volume filtered
Region (e.g. off Pacific Mexico)	Region (e.g. off Pacific Mexico)
Treated, R 1	**Treated, R 1**
Measure volume!	Measure volume!

dd.mm.yy	dd.mm.yy
Uptake	Uptake
Volume filtered	Volume filtered
Region (e.g. off Pacific Mexico)	Region (e.g. off Pacific Mexico)
Treated, R 2	**Treated, R 2**
Measure volume!	Measure volume!

dd.mm.yy	dd.mm.yy
Uptake	Uptake
Volume filtered	Volume filtered
Region (e.g. off Pacific Mexico)	Region (e.g. off Pacific Mexico)
Treated, R 3	**Treated, R 3**
Measure volume!	Measure volume!

E. coli
Write directly on Petri disc with waterproof pen.

Take a note and place this on your door or anywhere you see it frequently when incubation time terminates and the bacteriae dishes need to be analysed.

Uptake sampling

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Control

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated

Discharge sampling

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Control

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 1

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 2

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 3

Enterococci
Write directly on Petri disc with waterproof pen.

Take a note and place this on your door or anywhere you see it frequently when incubation time terminates and the bacteriae dishes need to be analysed.

Uptake sampling

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Control

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated

Discharge sampling

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Control

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 1

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 2

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 3

Cholera
Write directly on sample jar with waterproof pen. As the samples need to be shipped and the incubator may need to be opened at customs or security, do not write "Cholera" on any of the samples as this may cause trouble. Instead write C-water samples.

Uptake sampling

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Control

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated

Discharge sampling

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Control

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 1

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 2

dd.mm.yy
Uptake
Volume filtered
Region (e.g. off Pacific Mexico)
Treated, R 3

Annex 11 Set-up of sampling gear at the sampling point

The following outlines the working steps to take before the sampling may start, during sampling and after the sampling was completed.

This Annex should be printed and hang to the wall in the engine room.

Before the sampling starts

- Identify control and treated sampling line (ask vendor personnel)
- Place labelled buckets near sampling line outlets
- Place one thermometer at the bottom of each bucket
- Put wash bottle next to each bucket (check label)
- Put measuring cup to collect "continuous" drip sample next to each bucket
- Hang up HydroBios nets with ropes over water collectors
- Unscrew lid of cod-ends, put lid next to bucket (check label on lid) and screw cod-end to net
- **Check if cod-end valve is closed completely**
- Make sure that flow meter sealing rings are fitted and don't fell off
- Hang flow meters with top outlet tube extending into net and secure flow meter to net with rope
- **Check if flow meter was put back to zero** (if not, open transparent lid and press the left two bottoms simultaneously for two seconds)
- Check if flow meter inlet tube is properly fixed to sampling line, but don't connect yet with flow meter.
- When manipulating the flow meter valve substantial pressure may occur in the sampling line. Therefore it is preferred to have a double fixing ring screwed to the sampling line and flow meter inlet connection.
- Use tape to ensure that the metal fixing ring and screw cannot damage the net
- Note date and sample type, i.e. uptake or treated

During sampling

- After the water flow has started wait at least 2 minutes with the open flow meter valve (at the flow meter inlet tube) to wash the pipes. When doing so drain water into water collecting buckets underneath nets

- Check if the discharge of the water collecting buckets works
- Note start time of the sampling event
- Close flow meter valve completely and connect to flow meter
- Open valve and start feeding water into net. Minimize water spillage to the floor, i.e. make sure that the filtered water enters into the water collecting buckets underneath the net. If needed manipulate the position of the outlet tube of the flow meter to minimize water spillage
- Fill wash bottle with water filtered through net and put bottle into bucket
- Ask vendor personnel for likely duration of the sampling event and roughly calculate the frequency of measuring cups needed to fill bucket[23].
- Tell vendor personnel to give a 10 minute warning before the sampling event should be terminated
- Fill the measuring cup every few minutes with water from flow meter outlet and empty into bucket. Put lid back on bucket to avoid possible water spillage from neighbouring sampling line or dust etc to get into bucket. After sampling is completed at least 5 L of water should have been collected over the entire sampling time[24]
- Control and manipulate water flow with flow meter valve to avoid water collectors under the net to overflow. However, make sure that at least 1000 L of water are filtered through the net
- Measure water temperature in all buckets after thermometer was at least 10 minutes covered with water and record temperature

End of sampling

- Close valve of flow meter, disconnect inlet tube from flow meter, place it in water collector and open valve again
- Record end time of sampling
- Use wash bottle to clean net and put wash bottle back into bucket
- Carefully unscrew cod-end that only the upper connection (cod-end to net) opens

[23] Be prepared that the estimated duration of the sampling event is inaccurate. As a result make sure that you "oversample" in the beginning to guarantee that the 5 L are in the bucket when the sampling event comes to an end. This may be done either by a higher sampling frequency in the beginning or by filling the measuring cup more in the beginning and reduce the volume sampled towards the end.

[24] This water is used to measure TSS/POC, salinity, phytoplankton and bacteriae.

- Place cod-end carefully into bucket thereby allowing water to drain into cod-end via the filtering panels. Make sure that no sample is spilled into bucket.
- Screw cod-end lid on and drop cod-end into bucket
- Put lid on bucket and make sure lid sits tight
- Read and note water volume filtered from each flow meter
- Put flow meters into transport bag
- Take nets down and put back into net plastic bag and put all nets into transport bag
- Take ropes down and put into transport bag
- Carry all items back to cabin / laboratory

Annex 12 Sample transport reminder

Print this page and hang to the wall where you see it before you leave the vessel.

Take all samples from fridge and freezer with you.

After packing Styrofoam boxes, seal box with tape.